REMARKS

Claims 11-21 have been cancelled without prejudice to continued prosecution. Claim 1 has been amended to indicate that the retroviral packaging cell includes a retroviral vector and an exogenous nucleic acid encoding the growth factor, wherein the growth factor is displayed on the surface of the retroviral packaging cell, and wherein the retroviral vector includes a nucleic acid encoding the polypeptide. Support for this amendment can be found throughout the specification, including at page 5, lines 30-35, page 7, lines 7-17, page 16, lines 24-30, and page 17, lines 26-30 of the specification. Claims 2, 4, and 6-8 have been amended to correct typographical errors and for consistency with claim 1. No new matter has been added. Applicants respectfully request reconsideration and allowance of claims 1-8.

Rejections under 35 U.S.C. § 112

The Examiner rejected claims 1-8, 11, and 12 under 35 U.S.C. § 112, first paragraph, for lack of enablement. The Examiner asserted that the specification "does not reasonably provide enablement for practicing the claimed method wherein any and all quiescent cells are transformed" as "such cells could encompass allogeneic, xenogeneic, as well as autologous cells." The Examiner further alleged that "it is not clear whether the method can be used for transforming any and all quiescent cells, such as senescent cells, because the requirement of growth factors for different cell types is different, and some cells may require more than one growth factors [sic] for entering into a new cycle of mitosis." Applicants respectfully disagree.

The specification enables one of ordinary skill in the art to transform any quiescent cell. As indicated in the specification, "quiescent" refers to cells that are unlikely to enter mitosis within the next 24 hours in the absence of appropriate growth stimulus. See, for example, page 5, lines 12-14 of the specification. Exposure of quiescent cells to the membrane or surface bound growth factor, however, induces them to start dividing. See, for example, page 4, lines 10-13 of the specification. In contrast, senescent cells are not capable of entering the cell cycle in response to stimuli (e.g., growth factors). See, page 31 of Bacchetti Seminars in Cell & Developmental Biology 7:31-39 (1996) (copy enclosed). Thus, the present claims do not encompass transforming a senescent cell.

The Examiner also indicated that the claimed methods as instantly recited encompass a retroviral packaging cell line carrying any and all vectors, while the specification is only enabling for a packaging cell carrying a retroviral vector. Amended claim 1 recites that the packaging cell line includes a retroviral vector.

Since claims 11 and 12 have been canceled, the Examiner's arguments regarding transplantation of xenogeneic, allogeneic, or autologous cells and consequences of administering retroviral packaging cells to a mammal or patient will not be addressed.

In light of the above, Applicants respectfully request that the Examiner withdraw the rejection of claims 1-8 under 35 U.S.C. § 112, first paragraph.

Rejections under 35 U.S.C. § 102

The Examiner rejected claims 1-8, 11, and 12 under 35 U.S.C. § 102(b) as being anticipated by Luskey et al. (Blood, 80:396-402, 1992) for the reasons of record set forth in the Examiner's office action dated June 8, 2001. Specifically, the Examiner asserted that the Luskey et al. reference teaches that prestimulation of bone marrow with various cytokines, including stem cell factor, increases the retroviral-mediated gene transfer into murine hematopoietic stem cells. Applicants respectfully disagree.

Present claim 1 indicates that the retroviral packaging cell line includes an exogenous nucleic acid encoding the growth factor, wherein the growth factor is displayed on the surface of the retroviral packaging cell. In contrast, the Luskey et al. reference discloses adding growth factors to the prestimulation and coculture medium to stimulate retroviral-mediated gene transfer. Thus, the Luskey et al. reference does not disclose a method of transforming a quiescent cell using a retroviral packaging cell that contains an exogenous nucleic acid encoding a growth factor. Therefore, the Luskey et al. reference does not anticipate the presently claimed invention. Applicants respectfully request that the Examiner withdraw the rejection of claims 1-8 under 35 U.S.C. § 102(b).

Rejections under 35 U.S.C. § 103

The Examiner rejected claims 1, 5-8, and 11-12 under 35 U.S.C. § 103(a) as being unpatentable over Luskey et al. in view of Paul et al (U.S. Patent No. 5,736,387) for reasons of

record set forth in the previous office action dated June 18, 2001. Specifically, the Examiner asserted that the Luskey et al. reference teaches that prestimulation of bone marrow with various cytokines including stem cell factor increases the retroviral-mediated gene transfer of human ADA-encoding DNA into murine hematopoietic stem cells. The Examiner also asserted that the Paul et al. reference teaches a vector wherein IL-2 encoding sequences are fused at the N-terminal of envelope sequences of amphotropic murine retrovirus or of ecotropic murine virus. The Examiner alleged that it would have been obvious to one of ordinary skill in the art to transform the producer cells of Luskey et al. with the envelope fusion vector of Paul et al. Applicants respectfully disagree.

The combination of cited references does not teach or suggest transforming a quiescent cell by exposing the quiescent cell to a retroviral packaging cell, wherein the packaging cell includes a retroviral vector that contains a nucleic acid encoding a polypeptide and an exogenous nucleic acid encoding a growth factor, wherein the growth factor is displayed on the surface of the packaging cell.

As discussed above, the Luskey et al. reference does not teach or suggest a method of transforming a quiescent cell using a packaging cell that expresses a growth factor from an exogenous nucleic acid. Rather, the Luskey et al. reference teaches that gene transfer is enhanced in bone marrow cells that have been pre-stimulated with growth factors, then cocultured with packaging cells in the continued presence of growth factors. In the Luskey et al. reference, growth factors are supplied in both the prestimulation medium and the coculture medium. The presently claimed method, however, allows quiescent cells to be transformed without prestimulation. As described in the specification, transduction rates are improved when packaging cells that can deliver both a growth signal and a retroviral vector are used. See, e.g., page 25, line 15 through page 27, line 25.

The Paul et al. reference does not remedy the deficiencies of the Luskey et al. reference. The Paul et al. reference discloses retroviral particles that can be used to target particular cell populations. The retroviral particles contain retroviral vectors that encode a chimeric targeting protein (CTP), wherein the CTP is composed of a ligand moiety (e.g., a cytokine) and an uptake moiety (e.g., an envelope protein). Retroviral particles produced using such vectors display the CTP on their surfaces. In embodiments in which expression of the CTP is not desired in the

targeted cells, a packaging cell line that contains a nucleic acid encoding the CTP can be used to provide the CTP in trans such that the resulting retroviral particles contain CTP on their surfaces. The Paul et al. reference does not teach or suggest that quiescent cells can be transformed using retroviral packaging cells that contain both an exogenous nucleic acid encoding a growth factor and a retroviral vector encoding a polypeptide, wherein the growth factor is displayed on the surface of the packaging cell.

Using the retroviral vectors of Paul et al. in the method of the Luskey et al. reference does not render the presently claimed invention obvious. Again, in the method of Luskey et al., quiescent cells are transformed only after both pre-stimulating the cells with growth factors for 48 hours and co-culturing the cells with the packaging cell in the continued presence of growth factors. As the combination of the cited references does not teach or suggest contacting a quiescent cell with a retroviral packaging cell that contains a retroviral vector and an exogenous nucleic acid encoding a growth factor, wherein the growth factor is displayed on the surface of the packaging cell, and the retroviral vector encodes a polypeptide, the present claims are non-obvious. The Examiner is respectfully requested to withdraw the rejection of claims 1 and 5-8 under 35 U.S.C. §103.

The Examiner rejected claim 3 under 35 U.S.C. § 103(a) as being unpatentable over Luskey et al. in view of Paul et al. as applied to claims 1, 2, 4, 5, 7, and 8, and further in view of Lyman et al. (U.S. Patent No. 5,554,512) for reasons of record set forth in the previous office action dated June 18, 2001. Applicants respectfully disagree.

Claim 3 limits the growth factor of claim 1 to stem cell factor or PLT3 ligand. As discussed above, the combination of the Luskey et al. and Paul et al. references does not teach or suggest the presently claimed method for transforming a quiescent cell. The Lyman et al. reference teaches flt3-ligand as an isolated or homogenous protein, as well as host cells transfected or transformed with expression vectors that comprise a cDNA encoding flt3-ligand. The Lyman et al. reference does not remedy the deficiencies of the Luskey et al. or Paul et al. references as it does not teach or suggest that quiescent cells can be transformed using a retroviral packaging cell that contains both an exogenous nucleic acid encoding a growth factor and a retroviral vector, wherein the growth factor is displayed on the surface of the packaging cell, and the retroviral vector encodes a polypeptide. Thus, the combination of cited references

does not render the presently claimed invention obvious. In view of the above remarks, the Examiner is requested to withdraw the rejection of claim 3 under 35 U.S.C. §103.

CONCLUSION

Attached is a marked-up version of the changes being made by the current amendment.

Applicant asks that claims 1-8 be allowed. No extension fees or extra claims fees are due. Please apply any other charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

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Version with markings to show changes made

In the claims:

Claims 11-21 have been cancelled.

Claims 1-2, 4, and 6-8 have been amended as follows:

1. (Three Times Amended) A method of transforming a [population of] quiescent [cells] cell with a nucleic acid encoding a polypeptide [for treating a disease or disorder], the method comprising:

exposing [a population of] said quiescent [cells] cell in vitro to a retroviral packaging cell [expressing] , said retroviral packaging cell comprising a retroviral vector and an exogenous nucleic acid encoding a growth factor, [so that the] wherein said growth factor is displayed on the surface of [the] said retroviral packaging cell, [the retroviral packaging cell carrying a vector comprising the] wherein said retroviral vector comprises said nucleic acid encoding [the] said polypeptide [for treating the disease or disorder, wherein said vector does not encode said growth factor], and

wherein the surface bound growth factor induces [the] said quiescent [cells] cell to divide, so that the nucleic acid encoding [the] said polypeptide [for treating a disease or disorder] can incorporate into the genome of [the cells] said cell.

2. (Amended) The method of claim 1 wherein [the] said quiescent [cells are haematopoietic] cell is a hematopoietic stem [cells] cell.

4. (Three Times Amended) The method of claim 1 wherein [the] said retroviral packaging cell [line display] displays multiple growth factors.

6. (Twice Amended) The method of claim 5 wherein [the] said growth factor is expressed as a fusion with a viral envelope protein and is fused to the envelope protein via a cleavable linker.

7. (Three Times Amended) The method of claim 5 wherein [the] said retroviral envelope protein is viral envelope SU protein.

8. (Twice Amended) The method of claim 1 wherein [the] said retroviral packaging cell [line] further expresses nucleic acid encoding a receptor to target the cells to the bone marrow and/or an immunosuppressive factor so that the receptor and/or immunosuppressive factor are displayed on the cell surface.

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Telomere dynamics and telomerase activity in cell senescence and cancer

Silvia Bacchetti

Human somatic cells have a mortal phenotype that is under stringent genetic control and is dominant over unlimited proliferation. Reversal of this phenotype (immortality) may be required for tumour growth. Somatic cell division is accompanied by a decrease in telomeric DNA resulting in progressively shorter telomeres, a process that has been implicated in the control of the proliferative lifespan of cells. Conversely, telomere stabilization and expression of telomerase, the enzyme that elongates telomeric DNA, have been proposed as crucial to cell immortalization and tumour growth. This review discusses the data supporting this hypothesis, focusing on human cells, and addresses their implications for the management of cancer.

Key words: telomeres/telomerase/proliferative potential/senescence/cancer

The replicative lifespan of normal and malignant cells

Somatic cells from human and other vertebrates are capable of only limited proliferation when grown *in vitro*, a phenomenon termed replicative senescence.[1] Since proliferative capacity varies with tissue of origin, species lifespan and donor age, senescence is thought to reflect a functional decline that is also associated with aging of the organism (reviewed in ref 2). The senescent phenotype is genetically controlled and is dominant over proliferation,[3] indicating that it is a programmed rather than a default state. The onset of growth arrest is timed by a biological process, being dependent upon the number of divisions the cells have undergone rather than time in culture, and its establishment is mediated by activation of the G1/S checkpoint through the interaction of the p53- and pRB-dependent pathways.[4-7] Senescent cells become incapable of new rounds of DNA synthesis and mitosis, but retain metabolic activity and long term viability, and are characterized by a specific pattern of gene expression.[2,7]

The mortal phenotype of somatic cells may not be compatible with, and may indeed contribute to the suppression of tumour growth. Whether tumour cells are, or need to be immortal is still under debate, as a proper assessment of this property would require their culturing or successive passage in animals. On the other hand, evolution of the malignant phenotype generally requires accumulation of independent mutations in several genes, and tumour growth is likely to depend upon the balance between mutant clone expansions and cell death.[8] This pattern of development may conceivably exhaust the division potential of normal cells, making acquisition of cell immortality one of the essential and rate-limiting steps in malignant growth.

Telomeres and the regulation of cell lifespan: the telomere hypothesis

Eukaryotic chromosomes are capped at each end by specialized structures, the telomeres, which are composed of specific proteins (see article by T. de Lange in this issue) and of simple repetitive non-coding DNA, TTAGGG in humans and other vertebrates[9] (reviewed in ref 10). The size of telomeres is highly variable among vertebrates[10,11-14] but their precise length is unknown. Telomeric DNA lacks restriction enzyme sites, and it is cleaved from genomic DNA as part of terminal restriction fragments (TRFs) also comprising subtelomeric DNA[15-18] (Figure 1A). Variability in both the position of restriction enzyme sites and in the extent of sequence loss at different chromosome ends (Figure 1B), combined with asynchronous cell division, result in heterogenous TRFs. Telomere length is therefore most commonly reported as the average size of these fragments, determined by Southern hybridization, or is estimated from this value.[19,20]

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Telomeres are essential for chromosome stability and function: they protect chromosomes from degradation and fusion (reviewed in ref 10), contribute to the architecture of the nucleus[21] (reviewed in ref 22), and in some organisms play a role in gene expression through the phenomenon of telomere silencing (reviewed in ref 23). Telomeric DNA, being at the ends of linear molecules, fulfils the dual function of shouldering the loss of terminal sequences resulting from DNA replication by RNA-primed DNA polymerases[24,25] (Figure 1B) and of providing sites for *de-novo* elongation of the chromosome, and thus compensation for sequence loss, by the ribonucleoprotein enzyme telomerase (see article by G. Morin, this issue and below).

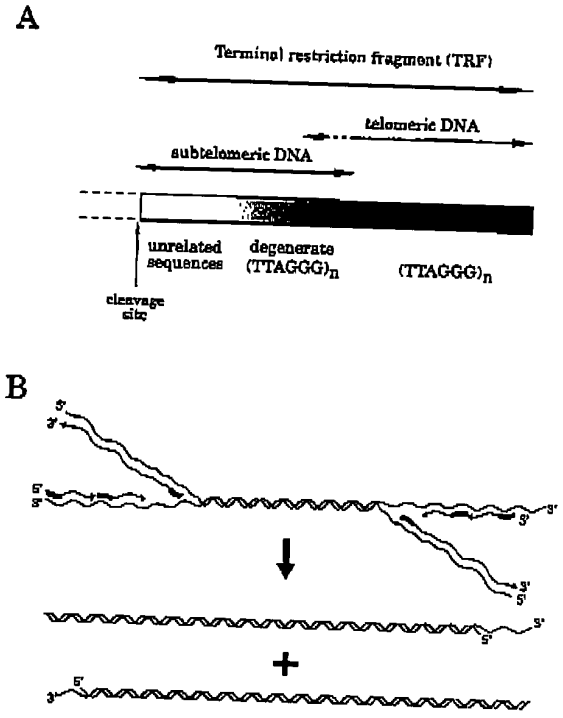

Figure 1. (A) Structure of terminal restriction fragments (TRFs). Digestion of genomic DNA with restriction enzymes releases TRFs comprising the telomere and a subtelomeric region. Telomeric DNA consists of perfect TTAGGG repeats; subtelomeric DNA contains degenerate TTAGGG repeats, possibly interspersed with perfect repeats, and unrelated repetitive sequences. (B) The end replication problem. DNA polymerases synthesize DNA in the 5' to 3' direction and require a primer, usually RNA, for initiation of synthesis. The diagram assumes initiation of replication at two internal origins near the ends of a linear molecule, but depicts only synthesis towards each end of the molecule. Following removal of primers and ligation of the Okazaki fragments, lagging strand synthesis yields daughter strands that are shorter at the 5' end than parental strands. The extent of sequence loss reflects the size and position of the 5' most primer; as the latter may vary, daughter molecules of different length may be generated.

It has been proposed that somatic cells are deficient in telomere maintenance and that loss of terminal sequences with each round of DNA replication is the process that records their proliferative history, while short telomeres provide the signal for growth arrest at senescence[25] (Figure 2). A corollary to this theory is that naturally immortal cells, such as sperm and ova, and cells immortalized by mutagenic events (e.g. malignant cells) should contain stable telomeres.[25] Since elongation of telomeric DNA by telomerase is the prevalent mode for telomere maintenance in eukaryotes (reviewed in ref 26 and by G. Morin in this issue), the activity of the enzyme may in turn be essential for cell immortality. The appeal of the 'telomere hypothesis' rests therefore in its providing a unifying molecular control for two phenotypically antithetic biological processes, aging and cancer.

Telomere shortening and cell senescence

The evidence in support of progressive diminution of telomere length in human somatic cells is indisputable, and comes from analysis of a variety of tissues and cultured cells. Unlike germline cells, which maintain telomeric DNA tracts of ≈20 kbp,[11,14,15,27-30] cells from postnatal somatic tissues contain telomeres whose size decreases with increasing donor age.[16,29-34] Studies with cultured somatic cells have further shown that loss of telomeric DNA is concomitant with, and dependent upon cell division and that telomere length is predictive of the cell proliferative capacity.[19,30,31,35]

Telomere dynamics in the human germline and soma generally correlate with intracellular levels of telomerase activity. Using a highly sensitive assay, based on PCR-amplification of telomeric repeats elongated by telomerase,[36] enzyme activity has been detected in germline cells but not in the majority of somatic tissues. Cells from negative tissues remain telomerase negative even when grown in culture,[19,31,36] indicating that their lack of activity *in vivo* is not due to a non proliferative state. To date, the only exceptions to a telomerase-negative adult soma are bone marrow and blood which have been found to express low levels of the enzyme.[37] Assay of cell fractions has revealed that in both tissues activity is

equally distributed among different cell types rather than being restricted to a subset of cells that may have self renewal capacity[38] (C-P. Chiu *et al*, submitted). This somewhat unexpected finding suggests that the presumed shut-off of the enzyme during development may not be absolute, and raises the possibility that telomerase activity may persist throughout the soma at levels below the sensitivity of current assays. The presence of the templating RNA of telomerase in normal somatic cells is compatible with this hypothesis, although it is unclear whether this component regulates levels of functional enzyme.[38a] Whatever the status and distribution of telomerase, it is apparent that enzymatic levels are insufficient for telomere maintenance in the soma, since telomeres decrease with cell division or aging in both telomerase positive and negative somatic tissues.

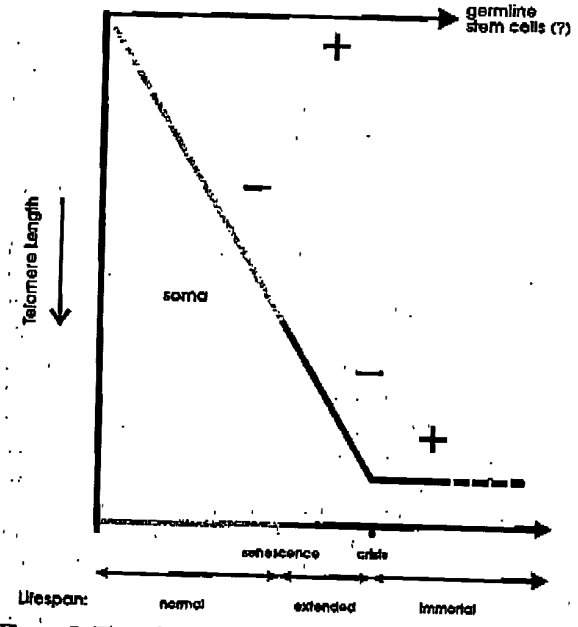

Figure 2. The telomere hypothesis. Reproductive cells, and perhaps stem cells, express telomerase activity (+) and maintain stable telomeres. At some point during development (– – –), telomerase is repressed (–) and, as a consequence, somatic cells lose telomeric DNA with each round of DNA replication and cell division throughout their normal lifespan. Shortening of telomeres is thought to monitor the proliferative history of somatic cells and to signal growth arrest (senescence) prior to critical loss of DNA and thus loss of cell viability. Transformation extends the proliferative capacity of cells but does not reactivate telomerase, and transformed cells continue to lose telomeric DNA until the end of their extended lifespan (crisis). At crisis, telomeres on average are extremely short and some may be absent, and the frequency of dicentric chromosomes increases. The loss of cell viability occurring at this stage may be caused by nonfunctional telomeres and/or lethal chromosome aberrations resulting from terminal fusion of unprotected chromosomes. The rare cells that reacquire telomerase activity have stable telomeres and are immortal.

Is telomere shortening of relevance to cell senescence? A causal link between the molecular and biological processes has yet to be established in human cells, and the correlative evidence accumulated to date is equally compatible with the two events being controlled by a common mechanism or being concomitant but independent. Several models have however been proposed to explain how shortened telomeres could signal growth arrest. The prevailing hypothesis[31,39] postulates that the progressive and likely asynchronous loss of telomeric DNA (Figure 1B) would ultimately generate one or a few unprotected chromosome ends. Such ends could activate the p53-dependent damage checkpoint by being detected directly as DNA breaks, or indirectly as aberrant chromosomes resulting from terminal fusion. This model is consistent with the presence of telomeric associations involving specific chromosomes in senescent cells,[40] and with the dependence of senescence upon expression of the p53 pathway.[4-7] Estimates of amounts of telomeric DNA at senescence[20,30,31,41] have been used to further support the existence of 'naked' chromosomes at this stage, but in the absence of precise measures of individual telomeres this postulate remains an *ad-hoc* feature of the model.

Since, after all, telomeres at senescence are still quite long[20,30,31,33] and of comparable average length in different cell types (TRFs ≈ 7 kbp; Figure 2), an alternative, and perhaps more likely signal for growth arrest may come from their general reduction to a functionally suboptimal size. It has been suggested, for example, that short telomeric DNA tracts may impair binding of telomeric proteins (see chapter by T. de Lange, this issue), resulting in abnormal chromatin and in telomeres that are incapable of normal associations among themselves and with the nuclear matrix.[10] The outcome may be aberrant segregation of chromosomes and thus activation of the p53 pathway. Interestingly, a chromatin domain with altered or non-nucleosomal pattern has been detected on short telomeres in transformed cells,[42] but it is not clear whether this domain is a constitutive feature of telomeres even in normal cells or whether its formation is strictly dependent on telomere length.[14] As discussed in the next section, models

invoking telomere-dependent karyotype alterations as a source of growth arrest signal must also be reconciled with the cytogenetic properties of cells driven experimentally to divide past senescence.[19,43]

An alternative to the damage checkpoint hypothesis, that also dispenses with the need for individual critically short telomeres, invokes silencing or derepression of senescence gene(s) with decreasing telomere length.[44] Since in yeast, where telomere silencing has been well characterized, the effect wanes as telomeres shorten,[45] activation of gene(s) that promote senescence would seem the most likely outcome of telomeric DNA loss. However, attempts to identify telomere position effects in human cells have so far been unfruitful[46] (T. de Lange, personal communication).

If senescence is indeed timed by telomere erosion, this mechanism may not be ubiquitous among eukaryotes. Mouse somatic cells senesce yet they do not appear to lose substantial amounts of telomeric DNA,[12,13,47] and in fact they are generally telomerase positive.[47,48] Similarly, unicellular organisms, such as yeast and Paramecium, undergo aging while maintaining telomere length.[49-51] Induction of telomere shortening in yeast and ciliates does result in growth arrest,[52-54] and the severity of the effect is magnified by extremely short telomeres.[52] However, growth arrest is accompanied by loss of cell viability, which suggests that the process may be more akin to the proliferative crisis experienced by transformed cells (see later) than to senescence.

Telomere stabilization and the immortal phenotype

A stronger argument than in the case of senescence can presently be made for a causal role of telomere dynamics in the immortal phenotype. *In-vitro* immortalized human cells maintain their telomeres[19,43,55-58a] and direct evidence that this process is essential for cell proliferation has recently been obtained. As with lower eukaryotes,[52-54] concomitant loss of telomeric sequences and of cell viability has been observed in an established cell line following inhibition of human telomerase by the antisense transcript of the template RNA.[58a]

Telomere dynamics in transformed but pre-immortal cells are also informative as to the role of these structures in the immortal and mortal phenotype. Transformation allows cells to bypass senescence and acquire an extended lifespan (Figure 2). Pre-immortal transformed cells continue to lose telomeres,[19,43,55-57,58a] indicating that maintenance of these structures is not a direct outcome of the transformation event. There are no obvious consequences of this protracted telomere loss until the population enters a proliferative crisis where, at variance with senescence, cell death generally occurs. At crisis, cells have telomeres which are on average several kilobases shorter than those of senescent cells (TRFs of ≈ 4 kbp versus ≈ 7 kbp), and accumulate a significantly larger number of dicentric chromosomes.[19,43] It has been proposed that cell death at this stage results from generalized loss of telomere function and/or lethal chromosome aberrations generated by telomere fusion,[19,43] a hypothesis now supported by more direct experimental analysis.[58a] Retention of viability and seemingly of telomere function during the extended lifespan, when telomeres erode below the size in senescent cells, has been explained by assuming that transformed cells can utilize imperfect telomeres (e.g. consisting of subtelomeric degenerate TTAGGG repeats intermingled with perfect repeats; Figure 1A) and lack the ability to recognize these damaged structures, or their byproducts, because of loss of damage checkpoints.[39,59] Mixed repeats mimicking the subtelomere are indeed capable of forming new telomeres in immortal cells, even if with reduced efficiency.[60] However, if unprotected chromosome ends exist at senescence, karyotype alterations should occur in transformed cells prior to crisis, and loss of checkpoints should contribute to their persistence. This is not necessarily the case,[19,43] most notably in EBV-transformed B lymphocytes which lose telomeric DNA but can retain a diploid karyotype until crisis. Minimally, this observation argues against telomere-dependent cytogenetic aberrations as a senescence signal, and is more compatible with the possibility that in senescent cells telomeres may still be present on all chromosomes and may be detected directly as damaged structures (e.g. breaks) or be non-functional.

Telomerase activity has not been detected in transformed human cells prior to crisis,[19,55,57,58a] except for EBV-transformed lymphocytes where, as in the case of the parental B cells, the PCR-based assay has revealed minimal levels of the enzyme in the pre-immortal phase (J. Gupta *et al*, unpublished). After immortalization telomere maintenance is most often accompanied by detectable[19,36,55] or elevated[43] levels of telomerase activity, a finding consistent with enzymatic elongation of telomeric DNA tracts. The temporal pattern of telomerase expression during cell immortalization suggests that the genetic instability

associated with short telomeres, by enhancing mutation rates, may be instrumental in activation or upregulation of the enzyme, or at least that critically short telomeres may drive selection for cells that express sufficient telomerase for telomere maintenance.[19,43]

Telomerase may be the most common but not necessarily the sole mechanism for the upkeep of telomeres in human cells. Several *in-vitro* immortalized cells that are telomerase negative have been described.[36,57,58a] As a population, telomeres in these cells are significantly longer than those usually seen in telomerase positive cells (TRFs ≈ 50 kbp versus 4–5 kbp) and appear stable. Analysis of individual telomeres has however revealed an alternating pattern of erosion and rapid elongation,[58] indicating that, paradoxically, telomere maintenance in these cells is concomitant with substantial telomere instability. These results are compatible with a recombinational mechanism for preservation of telomeres, as reported for yeast.[61,62] Of note, some telomerase negative immortal cells arise from populations that reach crisis with longer telomeres than those of cells that will acquire telomerase,[58a] suggesting that the putative recombinational mechanism may be activated or selected for independently of telomere length.

Lastly, telomere maintenance appears to be necessary for the immortal phenotype but may not be sufficient. Upon fusion of immortal cell lines, telomerase does not always cosegregate with immortality, and senescent hybrids that retain levels of enzymatic activity comparable to those of immortal siblings have been described.[58a] Although these findings may be restricted to hybrid lines, which undergo frequent chromosomal segregation, they provide a further caution against necessarily equating presence of telomerase activity with the immortal phenotype.

Telomerase: a tumour marker

There is now abundant evidence that tumour growth is associated with telomerase activity. Malignant tumours from essentially all tissues and sites have been assayed for the enzyme, using the PCR-based protocol[36] (reviewed in ref 63). Telomerase-positive tumours have been detected in all cases, and the positive fraction of tumours of a given type has often been found to exceed 90%. As a tumour marker, telomerase appears to be more common than p53 mutation, although the frequency of the latter event may be underestimated since other proteins can abrogate the p53 pathway (reviewed in ref 64). Interestingly, both of these prevalent markers contribute to carcinogenesis via a similar facilitating effect on malignant cell proliferation.

Based on the telomere dynamics of transformed human cells in culture, erosion of telomeres to a critically short size and the requirement for their stabilization (e.g. telomerase) are likely to be late events in the proliferative history of tumour cells. In terms of tumour progression however, they may occur at different clinical stages since the cell proliferative history will depend, among other factors, upon the fraction of dividing or dying cells as well as the cell age at onset and the genetic make-up of the malignancy (Figure 3). For example, cancers arising in the adult (i.e. in a founder cell with relatively short telomeres) or requiring a high number of mutations may most often present with short telomeres and telomerase activity at a relatively early clinical stage. Conversely, in childhood cancers and in those associated with fewer mutations, frank malignancies may consist of cells with long telomeres and lacking telomerase. Even in such cases, a mechanism for preservation of telomeres would ultimately be required for tumour expansion, progression to higher grade malignancy or recurrence. Lastly, benign growth and preneoplastic disease might generally be telomerase negative, and detection of the enzyme in preneoplasia may have both diagnostic and prognostic value.

The available data are in accord with these predictions.[63] No enzymatic activity has been detected in benign tumours,[36,65] whereas occasional samples of preneoplasias have been found positive.[36] In colorectal cancer, the best example of multistep carcinogenesis, essentially all adenocarcinomas are telomerase positive,[65] whereas in retinoblastoma, a developmental malignancy that may arise from one or two somatic mutations, the majority of tumours do not express detectable enzyme activity (J. Gupta *et al*, unpublished data). Enhanced telomerase activity, compatible with the expansion of a telomerase positive clone, correlates with disease severity in both leukemias and neuroblastoma[37,66] and, in the latter case, stage IVs tumours that regress spontaneously express low or no telomerase. At present however, too few temporal or longitudinal studies on sufficiently large cohorts of patients have been carried out for a proper assessment of the time-course of telomerase activation during tumourigenesis and of the diagnostic potential of this marker. Methods for assaying the enzyme or detecting enzyme components in single cells may be needed to resolve these issues.

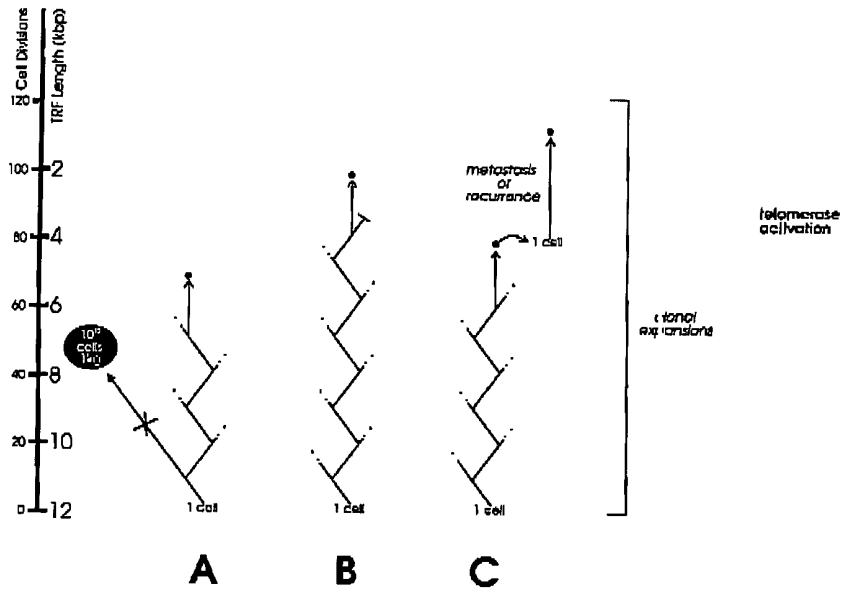

Figure 3. Telomere dynamics and telomerase activation during tumourigenesis. The proliferative capacity of normal cells is sufficient to generate a solid tumour that is clinically detectable (●) or even large enough to be physically lethal (e.g. 1 kg), if we assume exponential cell division and no cell death. This pattern of growth is unlikely given the genetic complexity of cancer. The requirement for accumulation of independent mutations in one cell entails expansion of mutant clones, most of which would abort (△) or be outgrown by best fit mutants. This process lengthens the proliferative history of malignant cells. Assuming TRFs of 12 kb in the original cell and the same rate of sequence loss estimated for cultural cells (≈100 bp/division), erosion of TRFs to 4 kb would require 80 cell divisions. At this stage selective pressure results in outgrowth of telomerase positive cells while telomerase negative cells die (↗) due to loss of telomere function. All other factors being equal, tumours may attain a detectable size without (A, C) or with (B) telomerase, depending upon the number of necessary mutations. It is assumed that telomerase activity will be an absolute requirement for expansion, progression or recurrence of tumours that may develop in the absence of the enzyme (C) (reprinted from ref 63).

Telomeres in human tumours

At variance with the nearly ubiquitous presence of telomerase in tumour samples, analysis of tumour telomeres has revealed extreme variability in length between and within tumour types (reviewed in ref 63). Although, by and large, telomeres are shorter in malignant than in control cells and their length decreases with tumour progression, there are numerous exceptions to this pattern and tumours with telomeres ranging from critically short to longer than those of control tissues have been described in nearly all types of cancer, whether positive or negative for telomerase. Since specimens were not always staged, and telomere stability was not assessed except in two studies,[67,68] the source(s) of this variability are unclear. An obvious role is attributable to the current methodologies that allow measures of telomeres only at the population level, but are capable of detecting telomerase in a few cells.[36] In addition, biological factors, such as presence of malignant cells with different proliferative histories, stabilization of telomeres at different lengths, telomere elongation after critical erosion, or contamination of the tumour biopsies with normal cells, are all potential contributors to telomere heterogeneity in tumours.

Whether malignant cells contain critically short telomeres is an issue that needs to be resolved since, as in the case of *in-vitro* transformed cells, these structures may have a dual and opposite effect on cell proliferation and hence on tumour growth. Through loss of function or generation of lethal chromosome aberrations, they could lead to cell death and tumour regression. As discussed below, the proposed use of

telomerase inhibitors in cancer therapy is based on this scenario. Conversely, short telomeres could contribute via generation of chromosomal rearrangements to the genetic plasticity of the cells and thus to the evolution of the malignant phenotype (reviewed in refs 69 and 70).

Telomerase and cancer therapy

The correlation between telomerase activity and the malignant phenotype has led to the proposal that inhibition of the enzyme may be exploited in cancer therapy,[56,57] and the finding that immortal cells undergo proliferative crisis when expressing the antisense template RNA of human telomerase[38a] lends support to the validity of this approach. Admittedly, the existence of telomerase positive normal cells together with the variability in telomere length among tumours, temper the earlier hopes that telomerase inhibition would have no side effects, and may restrict this form of treatment to those tumours that have short telomeres. In this case, the longer telomeres and lower division rate of normal cells should limit their loss of telomeric DNA, resulting in telomere 'aging' rather than cell death. Telomerase inhibitors would therefore act as selective, even if not totally specific, antitumour agents. In tumours where long telomeres are present, cytotoxicity of inhibitors would be restricted to the subset of cells with short telomeres, while cells with long telomeres would continue proliferating. A potential problem is that, depending upon the balance between these two processes, the outcome could be a cytostatic effect on tumour growth or even no initial effect. The resulting phenotypic lag would be clinically unacceptable and would demand alternative modes of intervention or a prolonged treatment which may ultimately lead to loss of telomeres in telomerase positive normal cells. Replacement of $(TTAGGG)_n$ with non functional sequences through overexpression of template mutants of the telomerase RNA[53] might circumvent this problem by acting in a telomere-length independent manner, but would affect telomerase positive normal and malignant cells with the same kinetics, unless targeted expression was achieved. In all of the above scenarios, however, it must be appreciated that telomerase inhibitors should still be less toxic than current chemotherapeutic agents, since they would target only those dividing cells that are telomerase positive rather than dividing cells at large. Thus, the use of such inhibitors in combination with other methods of tumour eradication remains a valid option.

A more critical issue that needs to be resolved in the context of a telomerase-based therapeutic approach stems from the existence of *in-vitro* immortalized cells that may have stable telomeres but no detectable telomerase activity.[58a,59,60] Telomerase-independent maintenance of telomeres may represent a salvage pathway that becomes operational even *in vivo* when telomerase cannot be reactivated or when telomerase activity is experimentally prevented, and precedents for this possibility have been described in yeast.[54,71]

Lastly, and irrespective of its use as a target for therapy, the potential role of telomerase as a diagnostic and prognostic marker merits investigation and adds to the hope that continued research on the enzyme may be of help in the management of cancer.

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